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Gene flow within and between regions: The population genetic structure of the phantom midge *Chaoborus crystallinus* (Diptera: Chaoboridae)

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Abstract

To determine the gene flow of *Chaoborus crystallinus*, populations throughout Europe were sampled. To test if the gene flow is higher within regions than between regions and to investigate if regional populations may act as metapopulations, four regions with several populations each were examined. For a detailed analysis of the regional gene flow, subregions within one region were analysed. Allozymes and mitochondrial restriction fragment length polymorphism (RFLP) were used to estimate the relative amounts of gene flow. On the European scale gene flow between populations within regions is higher than between regions. On the regional scale, gene flow between subregions is higher than between populations within subregions. Generally, the genetic differentiation between populations within regions is higher for the mitochondrial RFLP data than for the allozyme data. These results suggest that most of the dispersal is female dominated and mostly takes place between populations within regions. Due to this extensive regional gene flow, local adaptation may be influenced by regional processes. This might have interesting implications for the coevolution of the predator *C. crystallinus* and its plankton prey.

Keywords: Metapopulation; Ponds; Plankton; Genetic population structure; *Chaoborus*; *C. crystallinus*

Introduction

Several studies in the past have shown that freshwater pond systems are well suited as model systems for metapopulation studies and it has long been recognized that freshwater invertebrates are well suited for quantitative studies of dispersal (Bilton, Freeland, & Okamura, 2001; Bohonak & Jenkins, 2003). Surprisingly, studies on gene flow of active dispersal within freshwater metapopulations are still scares (Bilton et al., 2001). This is probably due to the fact that most studies, which have investigated the gene flow of invertebrates between ponds, focussed on passively dispersing organisms. Furthermore, for most of these organisms it remains unclear, if their population structure is comparable to a metapopulation setting. Metapopulations in their broadest definition are a group of populations, where each population is subject to stochastic extinctions and linked by dispersal (Haag, Hottinger, Riek, & Ebert, 2002; Hanski, 1999). Here we present a study on the phantom midge species *Chaoborus crystallinus* (Diptera: Chaoboridae) which lives predominantly in small ponds without

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fish. *C. crystallinus* is a dipteran and as with all dipterans *C. crystallinus* is holometabolous, and all larvae develop through four instars and pupate. From these pupae, imagines emerge and mate almost immediately. Imagines live only for 10 d at most, and the females do not feed during that period (Berendonk, 1999; Moore, 1986; Parma, 1971). Females lay a single egg raft in their lifetime (Berendonk, 1999; Borkent, 1979), and no resting stages are known. *C. crystallinus* is univoltine in Central Europe although it may go through two generations in exceptionally hot summers. In the past it has been demonstrated that, due to the restricted dispersal ability and the lack of resting stages, this species exists very close to the theoretical assumptions of a metapopulation (Berendonk & Bonsall, 2002). So far, no molecular methods were available to estimate gene flow for this species and only few data on its dispersal ability exist, which stem from direct estimates.

It has already been demonstrated that the flying ability of *C. crystallinus* is correlated with active dispersal behaviour and that long-range dispersal seems to be the exception (Berendonk & Bonsall, 2002). However, estimates of dispersal in freshwater insects, using direct observations of movements, are often problematic and the direct methods used in this past study, could only investigate a limited aspect of the dispersal of *C. crystallinus*. The use of genetic data provides an alternative approach to direct methods. Dispersal between populations results in the exchange of alleles (gene flow). Therefore, surveys of allele frequency (allozymes) or haplotypes (mitochondrial DNA) can be used to study dispersal. In simple models of population structure, the standardized variance in allele frequencies, calculated as Fst (Wright, 1951) or θ (Weir & Cockerham, 1984) is related to M(Nm), the effective number of migrants per generation. These models may be of limited relevance for metapopulations as local extinctions and founder effects could influence the above described relationship; however, such a signal of local extinctions should be detected using a comparison of nuclear and mitochondrial genetic variation.

We employed these methods to test the hypothesis that for *C. crystallinus* the gene flow between neighbouring ponds may be higher than between distant ponds. Results on the extent of the gene flow for local *C. crystallinus* populations would allow interpreations at what scale the local populations may act as a metapopulation. A confirmation of the above formulated hypothesis would therefore support the contention that local *C. crystallinus* populations within a region may be part of a metapopulation. This would be an important difference to passively dispersing plankton like *Daphnia* (Crease et al., 1997; De Meester, Gomez, Okamura, & Schwenk, 2002; Weider & Hobaek, 2003), where gene flow between neighbouring ponds is often as low as between distant ponds (Giessler, 1997; Straughan & Lehman, 2000). The above formulated hypothesis, that gene flow is higher within than between metapopulations, may apply to different geographic scales. Therefore, we investigated populations at a regional (Plön) and European scale. The extent of the geographic sampling scale was such that a comparison between the genetic population structures of *C. crystallinus* and other plankton organisms would be possible. Furthermore, we used different allozyme loci and mitochondrial DNA (ND4–ND5), which have substantially different properties. Allozymes are nuclear markers and have a relatively slow mutation rate compared with mitochondrial DNA, which is inherited primarily through the maternal line in insects (Rand, 2001).

Methods

Larvae of *C. crystallinus* live as plankton in the water columns of ponds. This facilitates their sampling. The larvae were caught, using a 30 cm diameter dip net and a 2.5 m pole. Larvae were collected from four different regions with several populations each, across the European continent. We sampled 12 populations in Plön (North Germany, belonging to four different "subregions"), five populations in the United Kingdom (Lake District), three populations in the Eifel area (Eifelmaare, Central Germany) and four populations in France (near Montpellier). The samples were taken within a radius of 100 km for all regions. The approximate geographic centres of the sampled areas are shown in Table 1 and Fig. 1. To consider the genetic variation within a region in detail, an extensive sampling in the region of Plön was conducted (12 populations, Fig. 1) and the populations within this region were assigned to subregions (four subregions). All collections were made in the same year during April and May before adult emergence, when 100% of the populations were confined to ponds as overwintering larvae. Hence 100% of the genotypes in the populations were available as larvae for sampling. Living larvae were transported to the laboratory and frozen at $-80\,^{\circ}$C so that genotypic samples were obtained directly from populations overwintering in the wild.

Allozyme phenotypes were discriminated using standard methods for cellulose electrophoresis (Hebert &

Table 1. Shown are the coordinates and geographically indicative descriptions of the sampled regions of this study

Region descriptor	Coordinates
Plön (North-Germany)	54°11′N–10°26′E
EIFEL (Central Germany)	50°06′N–06°45′E
Montpellier (South-France)	43°46′N–03°28′E
UK (Pond district)	54°20′N–02°57′W

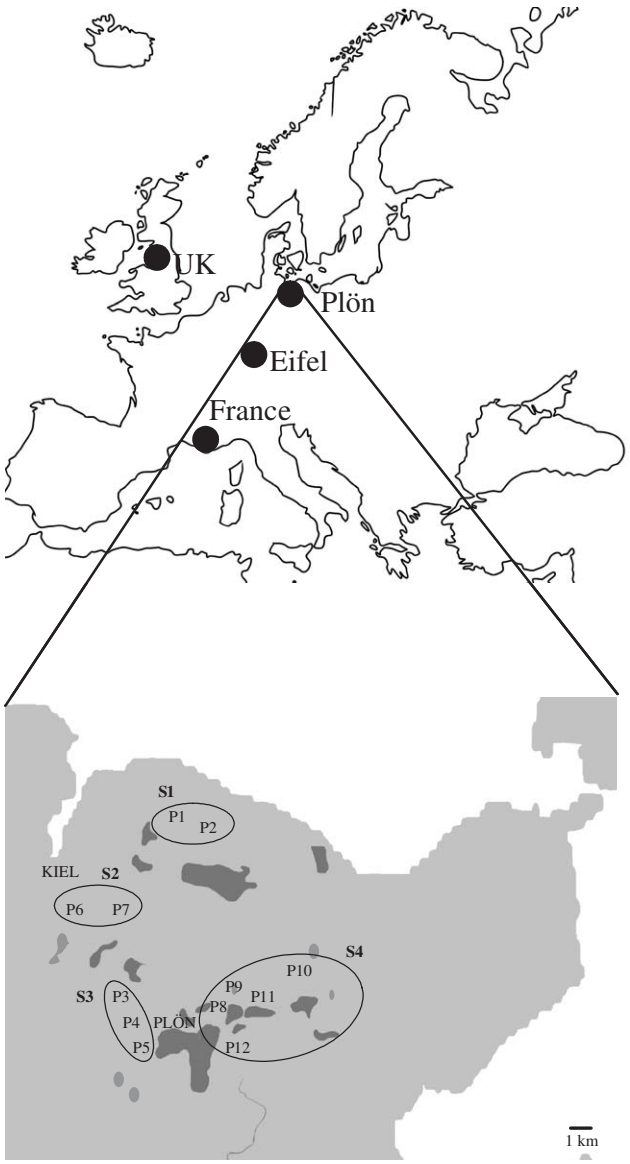

Fig. 1. The sampled regions within Europe and the sampled populations (P1–P12) and subregions (S1–S4) within Plön.

Beaton, 1993) and optimized for *Chaoborus* using methods from Loxdale, Castanera, and Brookes (1983); Richardson, Baverstock, and Adams (1986) and Easteal and Boussy (1987). Cellulose electrophoresis was performed for 10 allozyme loci using standard techniques, 45 individuals of each population were analysed. Fumarate hydratase (Fum: E.C. 4.2.1.2), glucose-6-phosphate isomerase (Gpi: E.C. 5.3.1.9), isocitrate dehydrogenase (Idh: E.C. 1.1.1.42), lactate dehydrogenase (Ldh: E.C. 1.1.1.27), malic enzyme (Me: E.C. 1.1.1.40), mannose-6-phosphate isomerase (Mpi: E.C. 5.3.1.8), leucyl-valin peptidase (Leu–Val: E.C. 3.4.13), phenylalanine–proline peptidase (Phe–Pro: E.C. 3.4.11), phosphoglucomutase (Pgm: EC 5.4.2.2), trehalase (Tre: E.C. 3.2.1.28).

A region of 4620 basepair length was amplified including parts of the mitochondrial genes NADH dehydrogenase subunit 6 (ND6), ND3 and the complete ND4, ND5 genes for *C. crystallinus*. The position of the primers and the amplified fragment were determined by an alignment to the published complete mitochondrial sequence of *Anopheles gambiae* (genbank acc. no.: L20934). We also tested the COI–COII region of *C. crystallinus*, but this region was not variable enough to be screened with restriction enzymes. The mitochondrial ND4–ND5 gene was PCR amplified in a total of 50 µl reaction volume containing 1 µl of 1:10 genomic DNA dilution 50 pmol primers (forward: 5′TCCTCAG AAAGTTATCTGTCCTCA3′, reverse: 5′GATTCCA TGAAATCCAGTTGC3′) 5 µl 10× reaction buffer, 200 µM nucleotide mix, and 1.5 units Taq polymerase (Promega). PCR conditions were: denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 5 min. Following evaluation of 5 µl PCR product from each reaction on a 1% agarose gel, the PCR products were purified using the Qiagen Qiaquick PCR Purification Kit. Cycle sequencing reactions were carried out using ABI's Dye Deoxy Terminator Cycle Sequencing Kit and samples were purified with an ethanol precipitation and analysed on an ABI 370 DNA Sequencing System. We sequenced part of the fragment to verify its identity as ND4ND5. Because the number of individuals was too high for all to be sequenced, we screened the PCR products of all populations (30 individuals of each population) with restriction enzymes. We chose the following nine enzymes: Nla III, Dra I, Mfe I, Rsa I, Hae III, Bfa I, Hpa I, Hpa II and Scrf I. The digests were evaluated on a 2.5% high-resolution agarose gel and the pattern of the digest was noted as presence/absence patterns, which resulted in a mitochondrial restriction fragment length polymorphism (RFLP) data set.

To examine the population differentiation, we performed an analysis of molecular variance (AMOVA) for the allozyme and the RFLP data using Arlequin (Schneider, Kueffer, Roessli, & Excoffier, 1997). The advantage of AMOVA lies in its analysis of variance in gene frequencies, while taking into account the number of mutations between molecular haplotypes. This means that for the calculation of the genetic variance, the genetic distances between haplotypes are included as well as the frequency of the respective haplotypes. Arlequin handles RFLP haplotypes of arbitrary length. Each restriction site is considered as a distinct locus. Furthermore, the statistical tests implemented in Arlequin are such that the hidden assumptions are minimized and are as powerful as possible. Thus, they often take the form of either permutation tests or exact tests. Three levels of subdivision were analysed: between geographical regions, within regions and within

populations. The four geographic regions and individual populations are those identified in Fig. 1 and Table 1. To test the significance of the variance components, nonparametric permutations were conducted: haplotypes were permuted within and among regions, and populations were permuted among regions. This procedure does not require an assumption of normality, nor does it require an assumption of equality of variance among populations. The haplotype and allele-frequency correlation measures produced in AMOVA are called Φ-statistics and are analogous to standard F-statistics (Wright, 1951). The population differentiation is presented as Φ_{ST} for the differentiation between populations within a region, Φ_{RT} as the differentiation between regions and Φ_{IT} as the differentiation between individuals within populations. We calculated the Φ_{ST} for all four regions. For a more detailed analysis of the regional genetic differentiation we applied the same calculations within the region Plön. To achieve this, all lakes, which clustered geographically close together, were assigned to four different "subregions": S1 (two populations), S2 (two populations), S3 (three populations) and S4 (five populations).

To determine patterns of isolation by distance, the relationship of population pairwise M (an estimate of Nm, number of effective migrants per generation) and k (pairwise geographic distance between populations) was evaluated (Slatkin, 1993). M values were generated in Arlequin (Schneider et al., 1997) using the relationship $M = (1 - F_{ST})/2F_{ST}$. We regressed $\log M$ against the log of the straight-line distance between populations. To evaluate the significance of the relationship between the two matrices, we computed a Mantel test statistics (Mantel, 1967) using Genepop (version 3.2a; (Raymond & Rousset, 1995).

Results

For the allozyme data, there was no evidence of a deviation from the expectation of Hardy–Weinberg equilibrium. While we cannot test directly for linkage, we can examine pairs of loci within populations for evidence of non-independence of genotypes. Such non-independence would suggest that linkage was important. In all, we performed 1265 tests and after applying a Bonferroni correction could not detect evidence of a linkage disequilibrium (z-value = 4.11, $p < 0.05$).

AMOVA revealed that, for the allozyme data, across the European continent, the degree of genetic differentiation between regions (Φ_{RT}) was 0.34, between populations within regions (Φ_{ST}) the value was 0.04. Furthermore, a large part of the genetic variation is observable within local ponds (ca. 63%, Table 2). Across the Plön region the genetic differentiation between subregions (Φ_{RT}) was 0.00 and not significant, between populations within subregions (Φ_{ST}) the value was 0.02. Almost all of the genetic variation was detected within local ponds (ca. 98%, Table 2).

For the mitochondrial RFLP data we were able to test for deviations from neutrality (Tajima's D, Tajima, 1996). In none of the populations did we find such a

Table 2. Analysis of molecular variance (AMOVA) for three levels in the hierarchy of *C. crystallinus* larval populations, d.f. = degree of freedom. The last column contains p-values, which indicate the statistical significance of the AMOVA

Source of variation	d.f.	Variance components	Percentage of variation	Fixation indices	Significance
EUROPE—*C. crystallinus*					
Allozyme					
Between regions	3	0.84	34.55	$\Phi_{RT} = 0.34$	**<0.001**
Between populations within regions	20	0.06	2.52	$\Phi_{ST} = 0.04$	**<0.001**
Within populations	2068	1.53	62.94	$\Phi_{IT} = 0.37$	**<0.001**
Mitochondrial RFLP					
Between regions	3	5.23	44.08	$\Phi_{RT} = 0.44$	**<0.001**
Between populations within regions	20	2.12	17.90	$\Phi_{ST} = 0.32$	**<0.001**
Within populations	696	4.51	38.02	$\Phi_{IT} = 0.62$	**<0.001**
PLOEN—*C. crystallinus*					
Allozyme					
Between regions	3	0.01	0.19	$\Phi_{RT} = 0.00$	>0.05
Between populations within regions	8	0.03	1.82	$\Phi_{ST} = 0.02$	**<0.001**
Within populations	1016	1.44	97.99	$\Phi_{IT} = 0.02$	**<0.001**
Mitochondrial RFLP					
Between regions	3	0.00	0.00	$\Phi_{RT} = 0.00$	>0.05
Between populations within regions	8	1.89	26.90	$\Phi_{ST} = 0.27$	**<0.001**
Within populations	348	5.23	74.50	$\Phi_{IT} = 0.25$	**<0.001**

deviation (no rejection of the null hypothesis of neutrality, $P > 0.05$). This test indicates that the amount of observed genetic variation within the haplotypes could be explained due to random mutations. For those data, the degree of population differentiation between regions (Φ_{RT}) was 0.44, between populations within regions (Φ_{ST}) the value was 0.32. However, in contrast to the allozyme data, a much smaller part of the genetic variation is observable within local ponds (ca. 38%, Table 2), and a substantial part of the variation can be detected between ponds within regions (ca. 18%). Across the Plön region the genetic differentiation between subregions (Φ_{RT}) was 0.00 and not significant, between populations within subregions (Φ_{ST}) the value was 0.27. Most of the genetic variation was found within local ponds (ca. 75%, Table 2).

The results of the isolation by distance (Figs. 2 and 3) show that with increasing geographic distance the gene flow decreases between populations. The Mantel test for the allozyme and mitochondrial RFLP data reveals that for both data the relationship is significant (Mantel test, $p < 0.05$, Figs. 2 and 3).

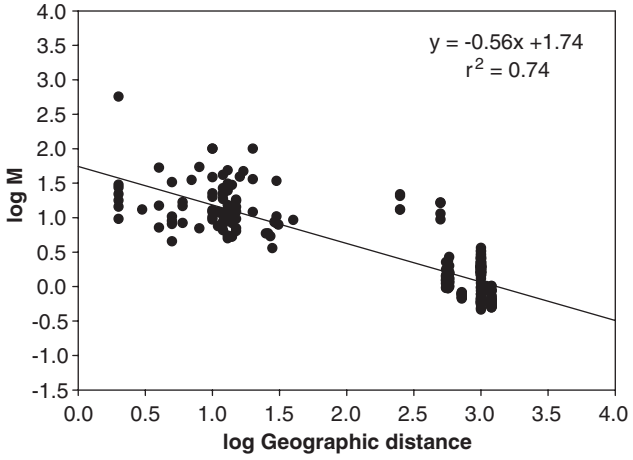

Fig. 2. The relationship between the gene flow measure (log M), and the straight-line geographic distance (log km) between each pair of sample sites for the allozyme data of *C. crystallinus* (Mantel test $p < 0.05$, $r^2 = 0.86$).

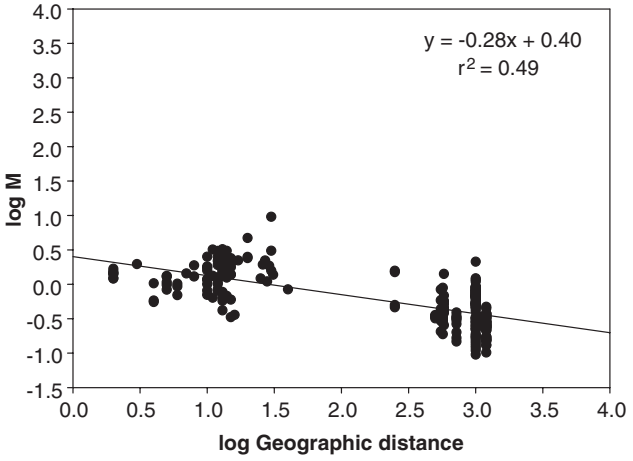

Fig. 3. The relationship between the gene flow measure (log M), and the straight-line geographic distance (log km) between each pair of sample sites for the mitochondrial RFLP of *C. crystallinus* (Mantel test $p < 0.05$, $r^2 = 0.60$).

Discussion

The hierarchical sampling scheme employed in this study allowed us to test the hypothesis that between neighbouring ponds, the gene flow of *C. crystallinus* will be higher than between distant ponds. The results in Table 2 show that, on the European scale, for both genetic markers the differentiation is considerably higher between regions compared to between populations within a region. This allows the conclusion that, on this geographic scale, the gene flow within regions is higher than between regions. However, the genetic differentiation between subregions is similar compared to the differentiation of populations within subregions on a small geographic scale (Plön). This suggests that the gene flow between close ponds is not higher than between distant ponds within this region. In summary, the results show that most of the gene flow takes place within regions and therefore it seems justified to conclude that the local populations of one region are part of one metapopulation. Especially, the results of the Plön region suggest that within regions local populations interact over a scale of approximately 50 km distance and are not substructured within regions. Here it is important to note that the total genetic variation might be somewhat inflated due to the fact that during the PCR no proofreading Taq was employed. However as this was the case for all samples, the relative differences of genetic variation, for example, between and within regions should not be influenced.

Interestingly, the values for the genetic differentiation of the populations within regions are much higher for the mitochondrial RFLP data than for the allozyme data. This is concordant with the fact that the effective population size for mitochondrial loci is only one-quarter compared to allozyme loci because of the maternal inheritance of mtDNA (Rand, 2001). However, the disparity between both genetic markers is highest within regions and the variation of the mitochondrial data between local ponds is much lower than the allozyme variation (Table 2). This suggests the following scenario: First, that the colonizers are gravid females; second, the result may be interpreted in such a way that founder effects are still observable and hence few females have found the population within a pond

and their progeny has rapidly spread within the pond. This is supported by the ecology of *C. crystallinus*: the males emerge before the females and the females need to mate soon after emergence as long as the genitalia are still soft (Berendonk, 1999). That founder effects can still be observed may conceivably explained by the frequent extinction of local ponds (Berendonk & Bonsall, 2002) and by the fast colonization ability of *C. crystallinus*. This is illustrated by the fact that, once a female has reached a pond it can lay up to 300 eggs, which will develop into second instars within a few weeks. Therefore, the local population is able to reach the carrying capacity of the pond fast and consequently, the progeny of new colonizing females have a much lower growth rate, or are preyed upon by the resident larvae. In this respect would the genetic differentiation between local populations within a region be similar to the priority effect of the Monopolization Hypothesis formulated by De Meester et al. (2002). However, it is important to note that for *C. crystallinus* this process seems to act at a regional scale, whereas the above-mentioned hypothesis relates to a global scale.

The result that the genetic differentiation of ponds within a subregions is as high as between subregions, suggests that ponds get predominantly colonized from females, which are not from the next nearest-neighbouring pond. Consequently, the high genetic differentiation is in agreement with the theoretical work of Wade and McCauley (1988) who focussed their attention on the effect of frequent and spatially uncorrelated extinctions. The key feature of their model is the sampling error introduced at the colonization stage. Localized population turnover will increase genetic differentiation as long as the number of colonizers is no more than twice the number of immigrants into existing demes. This condition holds if immigration is independent of patch occupancy; colonizers are then simply migrants that come across an empty patch. This seems a likely scenario for *C. crystallinus* as it is difficult to imagine how gravid females could identify an empty pond from the distance and Berendonk and Bonsall (2002) could not find a preferential oviposition by *C. crystallinus* for empty sites. In summary, the data allow the interpretation that colonization of ponds is dominated by inseminated females from ponds within the region, but not from the next nearest neighbours.

Interestingly, Figs. 2 and 3 show that for both genetic markers, the geographic isolation correlates significantly with the gene flow, but the slope of the regression is different. Such a pattern is expected to evolve over time between populations that exhibit a stepping-stone model of dispersal (Slatkin, 1993). Although there is a considerable scatter around the data, it is quite clear that between the geographically distant populations a lower gene flow exists than between the geographically close populations. The sampling design of this study was not intended for an isolation by distance analysis, for this the sampled ponds should have been equally distributed throughout the species range of *C. crystallinus*; therefore, the interpretation of this analysis is limited. Nevertheless, despite the scatter of the data, the results are different in comparison to other plankton organisms such as Cladocerans, which commonly show higher levels of genetic differentiation across short geographical distances (Bilton et al., 2001). However, these are all plankton species that rely on passive dispersal, which is consequently influenced by vectors that transport their propagules. The data allow the interpretation that the gene flow between ponds within a region is higher for *C. crystallinus* than for other planktonic organisms, for example, Cladocerans. For some *Daphnia* species the measured Φ_{ST} value, using allozymes, varied from 0.11 to 0.6 (Giessler, 1997; Michels, Audenaert, Ortells, & De Meester, 2003; Straughan & Lehman, 2000; Vanoverbeke & De Meester, 1997) and therefore the Φ_{ST} value of 0.04 in *C. crystallinus* is almost an order of magnitude lower and therefore the regional gene flow is much higher. Although the Φ_{ST} values for *C. crystallinus* are low, they are significant (Table 2). As these regions are distributed throughout Europe, the results seem quite robust.

The result of a limited gene flow between regions may seem surprising for a winged insect, but this result is in concordance with the data on other actively dispersing aquatic insects, such as Culicidae (Service, 1997), Odonata (Mcpeek, 1989), Caddisflies (Wilcock, Nichols, & Hildrew, 2003) or stoneflies (Hughes, Mather, Sheldon, & Allendorf, 1999). Most of these aquatic insects display limited dispersal rates. However, as Bohonak and Jenkins (2003) state in their excellent review: ''Oversimplifications regarding widespread, frequent dispersal by winged adults should be supplanted by conclusions specific to each taxon, little progress has been made toward a comprehensive answer to this question'', more data on gene flow in aquatic insects are necessary. This is also reflected by the surprising phenomenon, that except for a few studies on dragonflies (Mcpeek, 1989) and water beetles (Bilton et al., 2001 and references therein), most of the limited data on gene flow in aquatic insects stem from studies on stream dwelling insects. In contrast, data on gene flow in aquatic insects inhabiting stagnant water are rather rare.

The data suggest that colonization and migration within a region does not occur predominantly among neighbouring ponds within a metapopulation. This may have important implications for the genetic diversity of this species, because *C. crystallinus* exists in a classic metapopulation with frequent local extinctions. Generally, local extinctions should decrease the genetic variation of a species. However, the degree to which this diversity is decreasing depends crucially on the mode of recolonization (Pannel & Charlesworth, 1999; Whitlock

& Barton, 1997). If the colonists are derived from a single nearby population, then the decrease in genetic variation is fast; however, if colonists are derived from a pool of extant populations, the genetic diversity may not be reduced. As the data suggest that colonization does not take place from the next nearest-neighbouring pond, this may conceivably be the case for *C. crystallinus* and would be one of the few empirical cases, which support this theoretical hypothesis; however, further work on other *Chaoborus* species with different population structures is necessary and in progress to elucidate these first results.

The results clearly suggest that gene flow in *C. crystallinus* is highest within regions, whereas the results of other studies suggest that the gene flow for other planktonic organisms is at least as high between regions as within regions (De Meester et al., 2002; Michels et al., 2003, 2001). Most of these planktonic organisms are an important prey for *C. crystallinus* larvae and therefore this difference in genetic population structure between predator and prey will have interesting implications for the analysis of local adaptation and coevolution of *C. crystallinus* and its prey organisms.

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